Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility

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Objective: To determine whether reactive oxygen species in peritoneal fluid might be a factor in infertility.
Design: Prospective study.
Setting: Andrology laboratory and gynecology clinic at a tertiary care facility.
Patient(s): Women with endometriosis (n = 15) or idiopathic infertility (n = 11) who underwent laparoscopy for infertility. Patients undergoing tubal ligation served as controls (n = 13).
Intervention(s): Aspiration of peritoneal fluid.
Main Outcome Measure(s): Reactive oxygen species levels, presence of polymorphonuclear granulocytes, and leukocyte distribution in peritoneal fluid.
Result(s): Reactive oxygen species were present in the peritoneal fluid of patients with endometriosis, idiopathic infertility, and tubal ligation. Levels of reactive oxygen species did not show a statistically significant difference between patients with endometriosis and the control group in either unprocessed or processed (cell-free) peritoneal fluid, but did differ significantly between patients with idiopathic infertility and controls in processed peritoneal fluid. Polymorphonuclear granulocytes (>1 x 10⁶/mL) were not present in the peritoneal fluid of any patient. Macrophage concentrations of peritoneal fluid did not differ significantly between controls and patients with endometriosis or idiopathic infertility.
Conclusion(s): Reactive oxygen species in the peritoneal fluid may not affect fertility directly in women with endometriosis; however, they may have a role in patients with idiopathic infertility. (Fertil Steril® 1997;68:826–30. © 1997 by American Society for Reproductive Medicine.)

Key Words: Peritoneal fluid, reactive oxygen species, endometriosis, idiopathic infertility

Endometriosis-associated infertility remains one of the most frustrating clinical situations encountered by the gynecologist (1). Although endometriosis is a common diagnosis in infertile couples, there remains a great deal of uncertainty about the mechanism and treatment of infertility in these patients. In advanced endometriosis, tubal occlusion and tubo-ovarian adhesions may interfere mechanically with ovulation, ovum pickup, and ovum transport (2). However, infertility is difficult to explain in patients with minimal or mild endometriosis with no anatomic distortions (3).

Reactive oxygen species, such as superoxide anions, hydroxyl radicals, and hydrogen peroxide, have been implicated in the impairment of sperm quality in fertile men. Reactive oxygen species are produced by polymorphonuclear (PMN) granulocytes and abnormal spermatozoa in the semen, and seminal levels of reactive oxygen species of >10 x 10⁶ counts/min (cpm) can impair the fertilizing capacity of spermatozoa (4–6). The presence of reac-
tive oxygen species in the peritoneal fluid has not been reported.

Peritoneal fluid is thought to contribute to the tubal fluid, so changes in the peritoneal fluid in endometriosis may affect fertilization adversely by affecting sperm motility and function (7). Because there are no previous reports in the literature indicating the presence of reactive oxygen species in the peritoneal fluid, this study was designed to assess the levels of reactive oxygen species in the peritoneal fluid of patients with endometriosis, idiopathic infertility, and tubal ligation.

MATERIALS AND METHODS

Peritoneal Fluid Collection

This study was approved by the Cleveland Clinic’s Institutional Review Board. Peritoneal fluid was aspirated during laparoscopy from the anterior and posterior cul-de-sac in infertile patients with endometriosis (n = 15) and idiopathic infertility (n = 11) and in patients having tubal ligation (n = 13). All of the tubal ligation patients had children and served as controls; none of these patients had endometriosis at laparoscopy.

Idiopathic infertility in a couple was defined as failure to achieve pregnancy after 1 year of timed and unprotected sexual intercourse. These couples had ovulatory cycles as documented by luteal phase P, a normal semen analysis as defined by World Health Organization guidelines (8), normal hysterosalpingogram, and a normal laparoscopy.

All peritoneal fluid was aspirated carefully under direct vision to avoid contamination with blood. The peritoneal fluid samples were transported immediately to the laboratory in a sterile plastic container, and the volume of each sample was measured. All surgery was performed in the follicular phase of the menstrual cycle.

Peroxidase Staining Test for PMN Granulocytes

The presence of PMN granulocytes in all peritoneal fluid samples was determined with a peroxidase staining test (Endtz test), as described earlier (9). In brief, 20 μL of peritoneal fluid was placed in a 2-mL vial with 20 μL of phosphate-buffered saline (pH 7.0) and 40 μL of benzidine solution. The sample then was vortexed and allowed to sit at room temperature for 5 minutes. A 5-μL aliquot was placed on a Makler chamber (Sefi Medical, Haifa, Israel) and examined for cells that stained dark brown (peroxidase positive). The presence of >10⁶ white blood cells per milliliter was considered to be a positive test.

Reactive Oxygen Species Measurement

An aliquot of peritoneal fluid was centrifuged at 500 × g for 7 minutes. The supernatant was collected and is referred to as “processed peritoneal fluid.” Reactive oxygen species were measured in both unprocessed and processed (cell-free) peritoneal fluid samples with a luminometer (model LKB 983; Wallac Inc., Gaithersburg, MD). Ten microliters of 5 mmol/L luminol (5-amino-2,3 dehydro-1,4 phthalazinedione; Sigma Chemical Co., St. Louis, MO) prepared in dimethyl sulfoxide (Sigma Chemical Co.) was added to 500 μL of unprocessed or processed peritoneal fluid specimens and vortexed. Blackers, Whitten, and Whittingham (BWW) medium (Irvine Scientific, Santa Ana, CA) was used as a blank. Ten microliters of 5 mmol/L luminol added to 500 μL BWW medium was used as a negative control. Levels of reactive oxygen species were determined by measuring chemiluminescence in the integrated mode for 15 minutes and were expressed as counted photons per minute (cpm).

Distribution of Leukocytes

Smears of unprocessed peritoneal fluid from each group of patients were prepared and stained using Wright’s stain. Each slide was evaluated for macrophages, monocytes, neutrophils, and lymphocytes. A total of 200 cells was scored in each smear.

Statistical Methods

Data are presented as medians and interquartile (25% and 75%) ranges. Peritoneal fluid volumes and values of reactive oxygen species were compared between the experimental groups and the control group with the Wilcoxon rank-sum test, and P < 0.025 (with Bonferroni correction) was considered statistically significant. Values for reactive oxygen species between unprocessed and processed peritoneal fluid in the three groups were compared with the Wilcoxon signed-rank test. Spearman’s rank-order correlation was used to assess the relation between reactive oxygen species values and patient age and peritoneal fluid volume. One-way analysis of variance was used to compare the leukocyte distribution in the three patient groups. The SAS statistical software package (SAS Institute Inc., Cary, NC) was used to analyze the data.

RESULTS

Patient Age, Peritoneal Fluid Volume, and Presence of PMN Granulocytes

The results were not normally distributed in the control and patient populations and were analyzed by nonparametric tests.

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There was no significant difference in age between patients in the endometriosis group (median, 32 years; interquartile range, 29.3 and 35.8 years), the idiopathic infertility group (median, 33 years; interquartile range, 31.3 and 34.8 years), and the control group (median, 37.5 years; interquartile range, 33 and 41 years). The peritoneal fluid volume in the control group (median, 4.6 mL; interquartile range, 3.1 and 7 mL) was statistically significantly different from that in the endometriosis group (median, 10.5 mL; interquartile range, 7.6 and 18 mL; \( P < 0.01 \)), but not from that in the idiopathic group (median, 10 mL; interquartile range, 3.2 and 12.4 mL). Myeloperoxidase staining did not demonstrate \( >1 \times 10^9 \text{PMN} \) granulocytes in the peritoneal fluid of the control group or in patients with endometriosis or idiopathic infertility.

**Reactive Oxygen Species**

The patients with endometriosis were classified as stage I (\( n = 9 \)), stage II (\( n = 4 \)), stage III (\( n = 1 \)), or stage IV (\( n = 1 \)) according to the classification of endometriosis of the American Society for Reproductive Medicine (10). All of the patients with endometriosis, regardless of stage, were pooled into one group for analysis because stage of endometriosis showed no correlation with reactive oxygen species in unprocessed peritoneal fluid (\( r = -0.29, P = 0.28 \)) or in processed peritoneal fluid (cell-free) (\( r = -0.24, P = 0.41 \)). Women with idiopathic infertility, but not women with endometriosis, had elevated levels of reactive oxygen species in both unprocessed and processed peritoneal fluid.

The levels of reactive oxygen species in the unprocessed and processed aliquots for each group are shown in Table 1 and Figure 1. Levels of reactive oxygen species in unprocessed and processed peritoneal fluid from patients with endometriosis showed no relation with patient age (\( r = -0.06, P = 0.82 \); and \( r = -0.13, P = 0.67 \); respectively) or peritoneal fluid volume (\( r = -0.15, P = 0.58 \); and \( r = -0.27, P = 0.36 \); respectively). The age of the patients in the control group had a negative correlation with reactive oxygen species in processed peritoneal fluid (\( r = -0.68; P < 0.04 \)).

No correlation was seen between the volume of peritoneal fluid and reactive oxygen species levels in the unprocessed (\( r = -0.12; P < 0.67 \)) or processed specimens (\( r = -0.44; P < 0.14 \)). Reactive oxygen species in unprocessed or processed peritoneal fluid from patients with idiopathic infertility showed no correlation with patient age (\( r = 0.06, P = 0.84 \); and \( r = 0.08, P = 0.79 \); respectively) or peritoneal fluid volume (\( r = 0.43, P = 0.18 \); and \( r = -0.36, P = 0.35 \); respectively).

<table>
<thead>
<tr>
<th>Table 1 Reactive Oxygen Species Levels in Peritoneal Fluid of Women With Endometriosis or Idiopathic Infertility</th>
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<tr>
<td><strong>Group</strong></td>
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<tr>
<td>Tubal ligation (control, ( n = 13 ))</td>
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<tr>
<td>Idiopathic infertility (( n = 11 ))</td>
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<tr>
<td>Endometriosis (( n = 15 ))</td>
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*Note: Values are medians with interquartile range in parentheses.

* Idiopathic infertility versus controls in unprocessed peritoneal fluid, \( P = 0.06 \).
† Idiopathic infertility versus controls in processed peritoneal fluid, \( P = 0.01 \).
‡ Endometriosis versus controls in unprocessed peritoneal fluid, \( P = 0.94 \).
§ Endometriosis versus controls in processed peritoneal fluid, \( P = 0.92 \).

**Leukocyte Distribution**

Compared with the controls (14.5% ± 8.9%), the distribution of macrophages was similar in both patients with endometriosis (14.3% ± 7.8%) and idiopathic infertility (21.0% ± 12.1%) (\( P = 0.20 \)). Similarly, the distribution of monocytes, neutrophils, and lymphocytes in the patients with endometriosis and idiopathic infertility was not statistically significantly different from that in the control group.

**DISCUSSION**

The peritoneal cavity is lined with a single layer of mesothelium and contains both soluble and cellular components. Water, electrolytes, and many small molecules cross freely into the cavity. The peritoneal fluid normally contains many substances, including steroids, prostaglandins, cytokines, growth factors, inhibin, and cholesterol.

The stage of the menstrual cycle at which the peritoneal fluid is aspirated is important because large variations in the peritoneal fluid volume can occur. In our study, all patients were in the follicular phase of the menstrual cycle at the time of laparoscopy, when peritoneal fluid was aspirated.

Polymorphonuclear granulocytes are present in large numbers only in patients with pelvic inflammation (3). The macrophages may release chemicals that could interfere with fertilization by decreasing sperm penetration capacity (2). Sperm motility is reported to correlate negatively with peritoneal fluid volume when sperm are incubated with peritoneal...
fluid from patients with endometriosis (11). We observed a statistically significant increase in peritoneal fluid volume only in patients with endometriosis. The increased peritoneal fluid volume indicates that ectopic endometrial tissue may stimulate the peritoneum to secrete more, or to reabsorb less, peritoneal fluid.

Reactive oxygen species exert their cytotoxic effects by causing peroxidation of membrane phospholipids, which results in an increase in membrane permeability, loss of membrane integrity, enzyme inactivation, structural damage to DNA, and cell death (12). Reactive oxygen species have a toxic effect in reproductive functions, such as in sperm motility and the capacity for sperm-oocyte fusion (4, 5, 13–16).

Chemiluminescent probes allow measurement of reactive oxygen species. Luminal (5-amino-2,3 dehydro-1,4 phthalazinenedione) is a chemiluminescent probe that reacts with a variety of reactive oxygen species, including superoxide anions, hydroxyl radicals, and hydrogen peroxide. Both intracellular and extracellular reactive oxygen species are measured by this technique because luminal can permeate cell membranes (4, 17).

Our study describes the important finding that reactive oxygen species are present in the peritoneal fluid of normal women. However, these levels of reactive oxygen species in the unprocessed peritoneal fluid are statistically significantly higher (100-fold) than the levels observed in the seminal ejaculates of healthy normal men. Reactive oxygen species of \( \geq 10 \times 10^4 \text{ cpm} \) in human semen are considered abnormal and deleterious to sperm function (4).

In our study, all patients had statistically significantly higher values of reactive oxygen species in unprocessed peritoneal fluid than in the processed (cell-free) peritoneal fluid (Table 1). Reactive oxygen species were not statistically significantly elevated in patients with endometriosis compared with controls or patients with idiopathic infertility. There is a wide variation in the levels of reactive oxygen species produced by the peritoneal fluid.

Recently, we have found that levels of reactive oxygen species are statistically significantly elevated in the semen of men with clinical varicoceles. These men have white blood cell concentrations of \(< 1 \times 10^5/\text{mL} \) (unpublished observation). The reason for the elevated reactive oxygen species is unclear. In these patients, the presence of abnormal spermatozoa or low antioxidative capacity of the semen may be responsible for the elevated reactive oxygen species. Similarly in women, low antioxidant capacity may explain the elevated levels of reactive oxygen species in the processed (cell-free) peritoneal fluid.

The increase in macrophage number and concentration in the peritoneal fluid of patients with endometriosis has been reported (1, 3). Although the concept is well documented, it is not universally accepted. Some investigators found no increase in the macrophage concentration (3, 18, 19). We found no association between elevated levels of reactive oxygen species and macrophages in any of the study groups. In addition, the levels of granulocytes in our study were not elevated.

Polymorphonuclear granulocytes are not a major source of reactive oxygen species in the peritoneal fluid. The elevated levels of reactive oxygen species that we saw despite a similar macrophage distribution point to the fact that the macrophage theory originally proposed by Haney et al. (20), and recently called into question by the same investigators (21), needs to be reassessed.

Idiopathic infertility accounts for 3%–18% of all female infertility (22). In our study, the extracellular level of reactive oxygen species in the cell-free frac-

Figure 1 Comparison of reactive oxygen species values between unprocessed (A) and processed (B) peritoneal fluid from patients with endometriosis, patients with idiopathic infertility, and patients after tubal ligation. Each box plot covers the middle 50% of the data between the lower and upper quartiles. Central horizontal line represents the median and the vertical line indicates the range of data.

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tion was clinically more important because it can impair sperm in the vicinity. Levels of reactive oxygen species were statistically significantly higher in patients with idiopathic infertility than in controls. This difference indicates that high levels of reactive oxygen species may contribute to infertility in patients with idiopathic infertility. The elevated levels of reactive oxygen species in these patients suggest reduced antioxidants, and therefore, a reduced capacity to scavenge increased reactive oxygen species. Antioxidants such as vitamin E and glutathione can neutralize the toxic effects of reactive oxygen species (23–25). We speculate that antioxidant supplementation may be beneficial in scavenging the high levels of reactive oxygen species in patients with idiopathic infertility.

In conclusion, similar concentrations of PMN granulocytes and macrophages are seen in the peritoneal fluid of controls and of patients with endometriosis or idiopathic infertility. The high levels of reactive oxygen species in the peritoneal fluid may not cause infertility directly in patients with endometriosis but may contribute to infertility in patients with idiopathic infertility. A lack of antioxidant enzymes or low antioxidant capacity may be responsible for the increase in the levels of reactive oxygen species. Measurement of the total antioxidant levels in these patients is critical to our understanding of the link between reactive oxygen species and idiopathic infertility.

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REFERENCES